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COVER SHEET FOR FILING PROVISIONAL
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Date: April 22, 2004

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§1.53(c).

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TITLE OF THE INVENTION *(280 characters maximum)*
Methods for Identifying Chemical Modulators of Ras Superfamily GTPase Activity

ENCLOSED APPLICATION PARTS *(check all that apply)*

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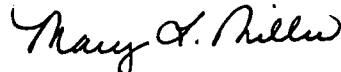Facsimile: (919) 854-1401
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METHOD OF PAYMENT

☒ Applicant claims small entity status. See 37 CFR §1.27.
☒ Check or money order is enclosed in the amount of $80.00 to cover the filing fee.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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Information for compound NSC#13778: (structure is attached as Figure 1)
3-(3-(dihydroxy(oxido)stibino)phenyl)acrylic acid:

This compound is part of the publicly available compound library
(>200,000 compound structures) from the National Cancer Institute. It
is part of a subset of compounds plated out for high throughput
screening called the Diversity Set (1,990 compounds) that can be
obtained for free from the NCI. This compound increases the rate of
Tiam1-mediated exchange on Rac1. No other small compound has ever been
reported to increase the rate of exchange on any GEF-GTPase pair.
We are currently elucidating the biochemical mechanism of
action of this compound.

Methods for identifying chemical modulators of Ras superfamily GTPase activity (UNC File No. OTD04-0018)
John Sondek and Rafael J. Rojas

Background: The Ras superfamily consists of a large number of related guanine nucleotide binding proteins (GTPases or G-proteins); of particular interest to cancer researchers and drug discovery groups are the Ras and Rho subfamilies due to their intimate role in cancer progression, cellular invasion, and cancer metastasis. GTPases act as molecular switches that cycle between ON and OFF states depending upon bound nucleotide. In the OFF state GDP is bound which renders the protein in an inactive conformation that is unable to activate downstream proteins. However, when GTP displaces the bound GDP, the GTPase can activate many downstream proteins by binding to effector proteins thereby leading to a physiological

Fig. 1 The guanine nucleotide exchange cycle. Ras superfamily GTPases are only active when bound to GTP. GEFs activate GTPases while GAPs inactivate GTPases. GDIs can sequester Rho GTPases and prevent activation.

response (see **Fig. 1**). Different classes of proteins regulate this exchange cycle between ON and OFF states. Guanine nucleotide exchange factors (GEFs) activate GTPases by allowing GDP to become displaced by GTP. For example, Tiam1 is a GEF that activates Rac. GTPase activating proteins (GAPs) accelerate the intrinsic GTPase activity of G-proteins, thereby rendering them inactive. Rho GTPases can additionally be regulated by guanine nucleotide dissociation inhibitors (GDIs), which prevent guanine nucleotide exchange. In particular, the Rho subfamily must retain the ability to cycle between ON and OFF states in order to reorganize the actin cytoskeleton. Due to their role in cancer and other diseases, Ras and Rho proteins are emerging targets for drug discovery; however, no current anti-cancer therapies target these oncogenic

proteins. Furthermore, no high throughput assays have been developed to identify inhibitors of these proteins.

We have recently developed a high throughput assay that can be used to identify compounds that modulate (inhibit or activate) the guanine nucleotide exchange cycle of Ras superfamily GTPases. The assay takes advantages of spectroscopic differences between bound and unbound fluorescent nucleotide analogs to monitor guanine exchange. Commercially available fluorophore-conjugated nucleotides have a low quantum yield of fluorescence in solution due to intermolecular quenching by solvent and intramolecular quenching by the guanine base. However, upon binding to G-protein, the fluorescence emission intensity from the fluorophore is greatly enhanced (see **Fig. 2**). This assay is
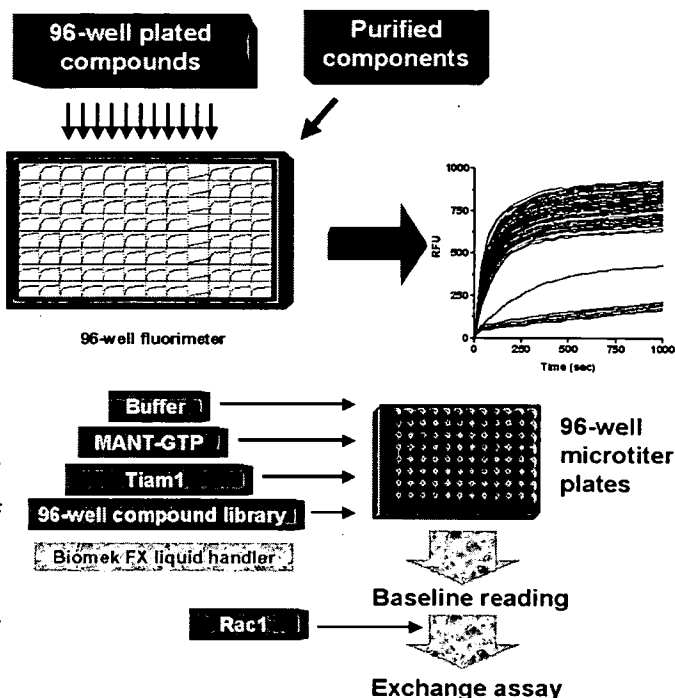
Fig. 2 High throughput, fluorescence-based guanine nucleotide exchange assay. The top diagram outlines the general scheme for screening with examples of output data, while the bottom diagram outlines components required for a screen. Components are added to a microtiter plate using a robotics liquid handler. Compound libraries can be screened for their ability to modulate the guanine nucleotide exchange reaction by monitoring fluorescence on a fluorimeter.

highly versatile because it can be used to discover compounds act via different mechanisms, all of which directly impact the nature of guanine nucleotide exchange. In this manner, the assay allows for discovery of compounds that can act on the GEF or the GTPase. Additionally, this assay can be utilized to discovery either inhibitors with decreased kinetics or activators with increased kinetics. The primary use for this assay would be drug discovery by using the assay for screening compound libraries. However, this assay can also be used

to rapidly characterize the biochemistry of any Ras superfamily GTPase and their regulatory proteins.

GEF inhibitors: Small molecule GEF inhibitors could be useful research tools to dissect signaling pathways mediated by these proteins. Additionally, compounds that target GEFs such as Tiam1 and Sos1 that are involved in disease states may be developed as a novel class of anti-cancer agents. Screening for GEF inhibitors would consist of monitoring GEF-mediated nucleotide exchange on a GTPase in the presence of a chemical from a compound library as outlined in **Fig. 2**. For example this assay can be used to screen libraries for compounds that inhibit Tiam1 (GEF) mediated activation of Rac1 (GTPase). Compounds that do not affect Tiam1 will catalyze exchange similar to control (black graphs in top panel of **Fig. 2**). However, compounds that inhibit Tiam1 will not have a large increase in fluorescence (red and blue graphs in top panel of **Fig. 2**). A secondary guanine nucleotide exchange reaction using an alternate GEF with the same GTPase can be used to show that the compound is GEF specific and does not alter the GTPase.

GTPase inhibitors: Screening for GTPase inhibitors can be conducted essentially as outlined above for identification of GEF inhibitors. These compounds will have a different mechanism of action because they will bind to the GTPase as opposed to the GEF. Compounds that inhibit the GTPase can be further divided into compounds that disrupt guanine nucleotide binding in the nucleotide binding cleft and compounds that bind to the conformationally sensitive switch regions thereby preventing interaction with the GEF. The data output as well as additional secondary assays can be used to readily distinguish the mechanism of action of any GTPase inhibitors discovered by this assay.

Effector inhibitors: Another potential application of our guanine nucleotide exchange assay is the discovery of small compound inhibitors of the effector-GTPase interaction. Effector proteins are the downstream signaling components that carry out the physiological response associated with GTPase activation. For example, a major downstream target for Rac is PAK, while Rho activates ROCK and Ras activates Raf. Small GTPases have an intrinsic rate of nucleotide

exchange that, although slow, can be effectively monitored using the fluorescence-based exchange assay. The binding of an effector protein to the GTPase, however, will reduce this spontaneous exchange rate dramatically. Over a period of time a GTPase will increase fluorescence intensity in a linear fashion due to spontaneous exchange. However, the addition of an effector protein or effector binding domain acts as a guanine dissociation inhibitor (GDI) preventing spontaneous exchange. By monitoring this increase in fluorescence in the presence of a compound library, and target effector protein, this assay can be used to discover compounds that disrupt GTPase-effector interactions.

Oncogenic Ras inhibitors: Ras is the most frequently mutated oncogene found in human cancers and is one the leading candidates for target-based drug design. Ras mutations render the protein resistant to RasGAP-mediated inactivation, thereby preserving the activated GTP-bound state. An emerging strategy for anti-cancer drug design is to inhibit activated Ras; one novel approach is to design compounds that allow mutated Ras to be inactivated by RasGAP. Our high throughput exchange assay can be modified to screen compounds for this purpose. By monitoring distinct fluorescent signals generated by GTP-bound and GDP-bound mutated Ras, we can screen large compound libraries for their inactivate mutated Ras.

GPCR screen: A vast majority of drugs on the market today work by modulating G-protein coupled receptor (GPCR) signaling. However, there are not many *in vitro*, fluorescence-based high throughput assays to screen for chemical modulators of GPCR signaling. Our high throughput guanine nucleotide exchange assay can be modified to screen purified GPCRs for chemical agonists. Activated GPCRs function as GEFs for G-protein alpha subunits. These G-proteins are significantly different from the Ras superfamily, however they still cycle between GTP-bound ON and GDP-bound OFF states much like Ras GTPases. By reconstituting the GPCR, G-protein alpha subunit system in lipid vesicles, we can monitor activation using the fluorescence-based guanine nucleotide exchange assay. This application has enormous potential for the rapid screening of novel high affinity ligands for many validated GPCR targets.

DESCRIPTION OF INVENTION

Ras superfamily GTPases are emerging drug targets that are intimately linked to the progression of cancer and acquisition of a metastatic and invasive phenotype. This superfamily consists of over 150 signaling molecules that activate a multiple of downstream effectors. The most extensively studied Ras superfamily GTPases are Ras, Rho, Rac, and Cdc42. These GTPases act as molecular binary switches that fluctuate between an active conformation (GTP-bound) and an inactive conformation (GDP-bound). Ras GTPases are able to interact with their cognate downstream effector proteins only when bound to GTP. Upstream signaling events in cancerous cells can lead to the activation of Ras GTPases by activating a class of proteins called guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP and allow binding of GTP. GTPase activating proteins (GAPs) inactivate Ras and Rho GTPases by increasing the intrinsic rate of GTP hydrolysis leading to accumulation of the inactive GDP-bound protein.

Ras is the most frequently mutated oncogene, found in over 30% of human cancers; similarly, activation of the Rho GTPases has recently been linked to metastasis and invasion of cancerous cells. Based on their link to the progression of cancer and the involvement in cancer invasion and metastasis, Ras and Rho GTPases are current drug targets for small molecule therapies. However, currently there is a lack of rapid high throughput methods for detecting chemical modulation of these proteins. The dependence on the discrete nucleotide bound states of Ras and Rho GTPases, coupled with the necessity for a GEF to initiate this activation has lead our group to develop numerous assays for Ras and Rho activation in solution.

Recently we have perfected an assay that can rapidly detect Rho GTPase activity in real time using fluorescence spectroscopy techniques. More importantly, this assay has been incorporated into a drug-screening platform that can detect chemical modulation of Ras and Rho GTPases in a high throughput manner. We have extensively refined this method for screening chemical modulators of Ras and Rho GTPases into its current form, which was recently used to identify the first small molecule activator of Rac signaling. After screening a small molecule library of 1,990 molecules (NCI Diversity Set) we have discovered one novel activator of Rac. Secondary studies have confirmed the activity found in the initial high throughput screen and ongoing studies are currently elucidating the mechanism of action of this novel small compound.

Our method for screening compounds is a novel modification of a well established method for detecting guanine nucleotide exchange on Ras and Rho GTPases based on the use of fluorescent guanine nucleotides, namely N-methylanthraniloyl (mant) conjugated guanine nucleotide (mant-GDP, mant-GTP). We have taken the previously described fluorescent nucleotide exchange assay and have adapted it for high throughput drug screening purposes. This novel adaptation for the use of drug screening is the basis for our invention. The high throughput exchange assay consists of 100 μL total volume containing: 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 40 mM Tris (pH 7.5), 400 nM GEF protein, 2 μM GTPase protein, 2 μM mant-GTP. Small molecules can be tested at concentrations up to 100 μM, with controls occurring in the presence of <1% DMSO. Ras and Rho GTPases are purified as previously described in the presence of GDP. Exchange factors for the GTPases Ras (Sos), RhoA (Dbs, Tim), Cdc42 (Dbs, ITSN), and Rac1 (Tiam1, Trio) are purified as previously described with constructs typically encoding for only the catalytic domains. We use an automated Biomek FX liquid handler robot to add all reagents to 96-well microtiter fluorimeter plates and conduct the assay in a 96-well

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compatible Gemini fluorimeter (Spectramax). The liquid handler is used to first add buffer components then, purified GEF protein, GTPase, and finally compounds. We use 96-well formatted small molecule libraries and test 80 compounds simultaneously in a single 96-well plate. We incorporate 16 control reactions into every 96-well plate that contains vehicle only (1% DMSO vol/vol). Once all reagents except for mant-GTP are added, we take an initial baseline fluorescence reading before the exchange reaction is initiated. All fluorescence readings are measured using fluorescence spectroscopy software SOFTmax PRO (Molecular Devices). Each well is excited using the endpoint fluorescence at 258 nm and with emission measured at 440 nm with a bandpass filter set at 420 nm. This baseline reading is subsequently subtracted from the kinetic reading for graphing purposes. After the baseline reading is completed, mant-GTP is simultaneously added to each well using the Biomek FX liquid handler to initiate the exchange reaction. GTPase activation is then measured in real time using the kinetic fluorescence setting while exciting at 258 nm and measuring fluorescence at 440 nm. The bandpass filter is set to 420 nm and the interval setting at 38 sec with 3 reads per well and 1 sec of mixing between fluorescent readings. Nucleotide exchange is monitored for 20-30 min and subsequently analyzed using Prizm software (GraphPad). All compounds with excessive (>2,000 RFU) or markedly reduced (<200 RFU) initial fluorescence are removed from further evaluation due to interfering fluorescence effects of the small molecule. Individual compounds are then analyzed for an increase or decrease in the catalytic exchange rate using the analytical software Prizm (GraphPad).

Rho family G proteins and cancer

Rho family G proteins coordinately control cytoskeletal rearrangements and transcriptional events necessary for various cellular process involving alterations in cell shape including: migration, cytokinesis, axonal growth, cellular polarity and differentiation. Like the majority of G proteins, members of the Rho family (typified by RhoA, Rac1, and Cdc42), cycle between inactive and active forms dictated by the state of bound guanine nucleotide. G proteins bound to GDP are inactive in downstream signaling while GTP-bound forms have increased affinity for a plethora of downstream effector proteins. Auxiliary proteins at several points normally tightly control cycling between GDP and GTP-forms. Dysregulation at any of these control steps can alter the regulated balance of active and inactive G proteins leading to aberrant signaling cascades and a variety of associated abnormalities.

GEFs for Rho family members are characterized by a tandem array consisting of a Dbl homology (DH) domain invariantly associated with an adjacent, C-terminal pleckstrin homology (PH) domain. Exchange activity predominantly localizes to DH domains while associated PH domains can regulate exchange activity by a variety of mechanism not completely understood. Aside from the universal requirement for associated DH and PH domains, Dbl-related GEFs vary widely in primary sequence and domain architecture, and comprise a large family of greater than 50 distinct eucaryotic members. Typically, overexpression or truncation of Dbl-related GEFs to remove auto-inhibitory sequences favors GTP loading onto various Rho family members. Consequently, many Dbl-related GEFs have been isolated based upon their tumorigenic potential in various cell-based assays. Clinically, chromosomal translocations leading to disruption of the Dbl-related GEFs bcr, abl, and LARG are associated with chronic myelogenous and acute lymphocytic leukemias while similar disruption of FGD1 lead to the developmental abnormality faciodigitogenital syndrome, also known as Aarskog-Scott Syndrome.

We are interested in understanding activation of Rho family G proteins by Dbl-related GEFs and using this information to predict and control associated cellular processes. To this end, we have recently solved crystal structures of several DH/PH fragments bound to their cognate Rho family G proteins depleted of guanine nucleotide (Tiam1/Rac1, Dbs/Cdc42, Intersectin/Cdc42). ~~██~~ These structures represent intermediates in the exchange process and present detailed, atomic resolution data on the conserved mechanism of exchange. Since Dbl-related GEFs show a wide range of specificities and promiscuities for Rho family G proteins, these crystal structures also present a detailed framework for understanding the various couplings between Rho-family G proteins and their associated GEFs.

In one embodiment, the present invention is directed to an of.. assay GEF activity using the loading and unloading of fluorescent analogs of GDP and GTP onto Rho family G proteins, ▬▬▬▬▬ employing an ▬▬▬▬▬ assay for high throughput screening (HTS) for compounds that interfere with the activation of Rho family G proteins by Dbl-related GEFs. Hits isolated from these screens would then be further characterized for efficacy in cell-based assays as exchange factor inhibitors. Ultimately, this research can provide lead compounds for inhibition of abnormally activated G proteins and the amelioration of associated pathologies, including cancer.

Exchange assays and HTS

Is used
a fluorescence-based assay to measure exchange activity of GEFs for various Rho family G proteins (▬▬▬▬▬) ▬▬▬▬▬ this assay to is modified develop HTS techniques useful for canvassing large numbers of compounds for specific inhibitors of guanine nucleotide exchange catalyzed by Dbl-related GEFs.

Our current fluorescence-based assays were modified from existing literature with an emphasis on simplicity and used GDP and GTP derivatized at the 2' or 3' hydroxyls with methyl-anthronyl (MANT). The fluorescent quantum yields of MANT-guanine nucleotides dramatically increase upon binding to G proteins, and this characteristic provides quantitative, real-time measurements of bound vs. free nucleotide. Typically, exchange reactions are performed in 2 ml cuvettes with limiting concentrations of MANT-guanine nucleotides relative to G protein. Spontaneous exchange of MANT-guanine nucleotides onto Rho family G proteins is relatively slow and significant loading of the fluorescent guanine nucleotides with consequent increases in fluorescence is initiated by the addition of catalytic amounts of GEF. The assay is extremely straightforward, requires no washing, filtering, or removal of reagents, and yields reproducible initial rates of loading and unloading of guanine nucleotides bound to G proteins. Furthermore, Cdc42, RhoA, and Rac1 loaded with MANT-nucleotides are stable for several hours under the reaction conditions, fulfilling an obvious requirement of HTS when sample preparation and fluorescent measuring are time-consuming.

While these assays are adequate for the routine analysis of distinct GEFs with specific Rho family G proteins, several parameters are unacceptable for HTS and have been ▬▬▬▬ modified. We will convert the current cuvette format to 96-well microtiter plates using a SpectroMax Gemini spectrofluorimeter plate reader (Molecular Devices) with dual monochromators for independent excitation and emission wavelength selection. ▬▬▬▬▬ compounds to be screened will be diluted into DMSO, added to duplicate wells of a microtiter plate, and dried. Subsequently, GEFs will be added and allowed to incubate with compounds before addition of G proteins. Prior to addition of fluorescent nucleotides, plates will be scanned and wells with excessive autofluorescence will be recorded as potentially confounding to subsequent analysis. Guanine nucleotide exchange will be initiated with the addition of MANT-GTP that will be allowed to incubate before reading. If necessary to allow post-reaction manipulations, exchange will be significantly quenched by the addition of millimolar concentrations of Mg2+ that stabilizes bound guanine nucleotide and incubation at 4oC. Efficacious inhibitors of GEF-catalyzed exchange are anticipated to significantly reduce overall fluorescence.

Our current techniques rely upon prompt fluorescence defined as the direct measurement of emitted fluorescence after excitation at a specific wavelength. However, while prompt fluorescence can be used for HTS, several alternate techniques ▄▄▄▄▄▄▄▄will be explored. For instance, fluorescence anisotropy (FA) measures changes in fluorescence polarization due to molecular motion. This technique is inherently ratiometric and is therefore less prone than prompt fluorescence to inner filter effects and autofluorescence from additional components introduced during screening of chemical libraries. Consistent with published results, the binding of MANT-GDP to Rho family G proteins (~20 kDa) represents a large change in the effective size of the fluorescent marker. Therefore tumbling rates and associated anisotropies of MANT-GDP are expected to be significantly altered upon binding G proteins, thereby allowing sensitive monitoring of exchange events. ▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄we will also explore the use of intramolecular resonance energy transfer using guanine nucleotides derivatized with BODIPY at the 2' or 3' hydroxyls. Unbound BODIPY-guanine nucleotides are internally quenched due to the interaction of the fluorescent group with the guanine base. Upon interaction with G proteins, the guanine nucleotide base is sequestered from solvent and inaccessible to the BODIPY derivative resulting in a large increase in the fluorescent quantum yield of BODIPY. Since internal quenching serves to reduce background fluorescence, we anticipate exchange techniques using BODIPY derivatives will produce significant improvement in signal to noise relative to similar MANT derivatives.

Specific GEF/G protein systems

RhoA, Rac1, and Cdc42 are typically studied as representative members of the Rho family of G proteins responsible for distinct morphological alterations in response to unique extracellular signals. We are currently studying several Dbl-related GEFs with varying activity toward these Rho family members. For instance, Tiam1 possesses exchange activity only toward Rac1, while intersectin is specific for Cdc42, and Dbs will exchange on both RhoA and Cdc42. Our on-going characterization of these mammalian GEFs provides us with a spectrum of exchange activities to profile against any hits derived from HTS ▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄▄ ▄▄most HTS will involve Tiam1 prior to complementary studies with either Dbs or intersectin.

Tiam1, or T-cell lymphoma invasion and metastasis factor 1 was originally identified by its ability to promote invasiveness of a normally non-invasive T-cell lymphoma. Similarly, Tiam1 promotes metastasis of these clonal cell lines upon injection into nude mice. Dbs was also isolated based upon its transforming potential while intersectin couples proto-oncogenic Ras to the Rho family of G proteins.

Our extensive and on-going characterization of Dbl-related GEFs provides unique opportunities to develop HTS techniques for development of lead compounds designed to inhibit a central control point of G protein activation that upon dysregulation promotes cancer.

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Background and Rationale

Various techniques following the fluorescent properties of reporter ligands and enzymatic substrates are readily amenable to modification for high-throughput screening in microtiter format. Given a multitude of advantages related to cost, speed and reliability, these techniques are rapidly replacing more conventional assays employing radioactivity or the physical separation of components. Fluorescent analogs of guanine nucleotides are now routinely used to study activities that alter the affinities and selectivities of G proteins for guanine nucleotides. We propose modifying existing fluorescence techniques to develop high-throughput screening (HTS) formats useful for assessing the functional binding properties of various G proteins for guanine nucleotides. Finally, these HTS techniques will be used to screen chemical libraries for compounds that modulate G protein activation for potential therapeutic use.

Specific Aims and Objectives

1. We routinely employ fluorescence-based assays to measure guanine nucleotide loading and unloading of Rho-family G proteins catalyzed by Dbl-family guanine nucleotide exchange factors (GEFs). These cuvette-based assays will be modified to use microtiter plates suitable for HTS. Subsequently, chemical libraries will be screened for compounds that inhibit the exchange of guanine nucleotides bound to several Rho-family G proteins and catalyzed by Dbl-family GEFs.

2. The application of fluorescent analogs of GDP and GTP to non-radioactive assays for G-protein coupled receptor (GPCR) activation of heterotrimeric G-proteins also will be investigated. Specifically, fluorescence-based, functional assays for cannabinoid CB-1 receptors (with possible extension to chemokine CCR5 receptors) will be developed to determine applicability of this technology to GPCR membrane preparations for future utility in drug screening and/or secondary drug testing.

Research Plan

We will convert the current cuvette format to microtiter plates using a SpectroMax Gemini spectrofluorimeter plate reader (Molecular Devices. HTS techniques require sufficient signal to noise, reproducibility, and time-independence to produce useful data. These variables will be optimized by varying experimental parameters including concentration of proteins and nucleotide analogs, buffer conditions, and temperature will be modified. If necessary, the order of reagent addition and type of fluorescence measured will also be examined.

Specific systems to be studied include the loading of guanine nucleotide onto Rac1 and Cdc42 catalyzed by the Dbl-family GEFs, Tiam1 and Dbs, respectively. ▓▓▓▓▓▓▓▓▓▓▓▓▓▓ all proteins will be heterologously expressed in *E. coli* and purified to homogeneity prior to characterization.

Once optimized conditions for HTS are determined, protocols and reagents will be ▓▓▓▓▓▓ used for ▓▓▓▓▓▓▓▓▓ screening of chemical libraries for compounds that inhibit GEF-catalyzed nucleotide exchange of Rho-family G proteins.

Similar analysis will be undertaken for the characterization of the GPCR systems.

Established and Emerging Fluorescence-Based Assays for G-Protein Function: Ras-Superfamily GTPases

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Abstract: Ras and Rho GTPases are signaling proteins that regulate a variety of physiological events and are intimately linked to the progression of cancer. Recently, a variety of fluorescence-based assays have been refined to monitor activation of these GTPases. This review summarizes current fluorescence-based techniques for studying Ras superfamily GTPases with an emphasis on practical examples and high-throughput applications. These techniques are not only useful for biochemical characterization of Ras superfamily members, but will also facilitate the discovery of small molecule therapeutics designed to inhibit signal transduction mediated by GTPases.

Keywords: Fluorescent nucleotide exchange assay, Ras, Rho, small GTPase.

INTRODUCTION

Ras-Superfamily GTPases: Molecular Switches of Signal Transduction

The Ras-superfamily consists of over 100 members categorized into several subfamilies based upon sequence homology. The Ras, Rho, Ran, Rab, Arf, and Rem/Rad family proteins are monomeric GTP hydrolyzing proteins (GTPases or G-proteins) of ~21 kDa essential for a variety of biological phenomenon (reviewed in [1-4]). Similar to their large heterotrimeric G-protein α-subunit counterparts, small GTPases act as binary switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. The GTP/GDP cycling of Ras superfamily GTPases, shown in Fig. (1), is highly regulated by classes of proteins specific to each subfamily. Guanine nucleotide exchange factors (GEFs) activate GTPases, while GTPase activating proteins (GAPs) cause inactivation. Small GTPases can additionally be regulated by guanine nucleotide dissociation inhibitors (GDIs) that prevent nucleotide exchange, sequester GTPases, and block associated downstream signaling.

Members of the Ras and Rho subfamilies are the most extensively studied group of small GTPases and are essential components of the mitogenic signal transduction pathway. Extracellular stimulation of receptor tyrosine kinases, G-protein coupled receptors, or integrins can result in activation of Ras and the prototypical Rho subfamily members RhoA, Rac1, and Cdc42 [5, 6]. Once activated, these GTPases further propagate external signals by activating a multitude of downstream effector proteins, resulting in a diversity of cellular responses. Additionally, activation of Ras and Rho family GTPases is a critical step during tumor progression and acquisition of an invasive and

metastatic phenotype [7-11]. These GTPases are highly oncogenic with over 30% of all human cancers and 90% of pancreatic cancers harboring activated Ras mutations [11]. Recent evidence also suggests a vital role for Rho family members during transformation and the acquisition of an invasive and metastatic phenotype by regulating the actin cytoskeleton [12-15]. Furthermore, GEFs specific to Rho GTPases (RhoGEFs) such as Dbl (diffuse B cell lymphoma), Tiam1 (T-cell invasion and metastasis factor), and LARG (leukemia associated RhoGEF) are routinely isolated during screens for transforming oncogenes and make up one of the largest classes of human proto-oncogenes with over 60 members (reviewed in [16, 17]).

It is due to their inherent oncogenic signaling properties and role in cancer metastasis that Ras and Rho-mediated signaling events are emerging targets for anti-cancer drug discovery [18]. Furthermore, the complex pathways that regulate Ras superfamily GTPases are the current focus of intensive research. For many of these pursuits there is a critical need for sensitive, real-time measurements of GTPase activation and subsequent monitoring of signaling events. Recently, a variety of fluorescence-based assays have been refined to monitor these events using purified proteins *in vitro* as well as under more physiological conditions *in vivo*. This review will summarize current fluorescence-based techniques for studying Ras superfamily GTPases and will illustrate practical examples of applications demonstrating their utility. Such techniques are useful not only for the biochemical characterization of these signaling proteins, but will also be promising for future strategies in target-based drug design for the treatment of cancer.

Anatomy of a Small GTPase

The structural elucidation of a number of Ras superfamily members including Ras [19, 20], Rac [21], Rap [22], and Ran [23] has been instrumental in revealing a universal mechanism for nucleotide binding, GTP

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Guanine nucleotide exchange factor
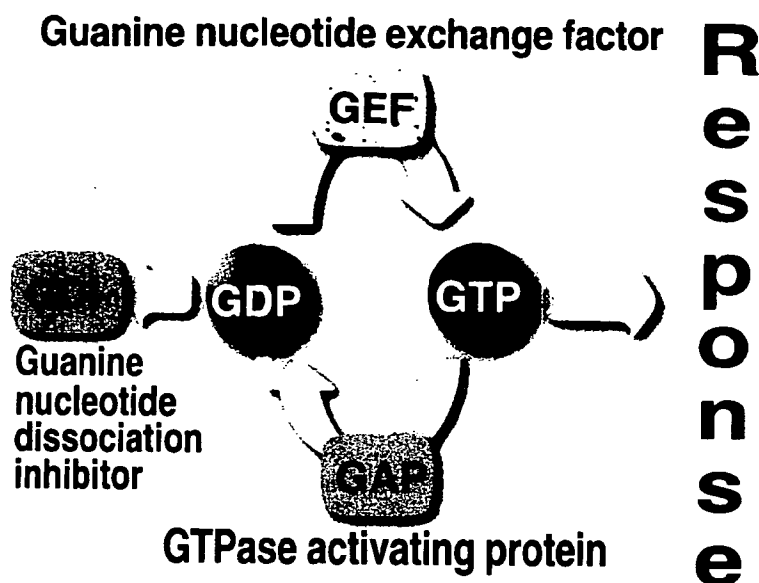
Fig. (1). The guanine nucleotide exchange cycle of small GTPases. Ras superfamily GTPases act as molecular switches that cycle between GDP-bound "off" state and GTP-bound "on" states. Guanine nucleotide exchange factors (GEFs) activate small GTPases, while GTPase activating proteins (GAPs) promote inactivation. Guanine nucleotide dissociation inhibitors (GDIs) prevent the loss of nucleotide and function to sequester G-protein from the nucleotide exchange cycle. Activated GTPase interacts with downstream effector proteins leading to various physiological responses.

hydrolysis, and conformational alterations associated with the state of bound nucleotide [24]. This structural knowledge has been applied to develop effective fluorescence-based assays to monitor these events. Small GTPases bind guanine nucleotides in a structurally well-defined nucleotide-binding pocket that uses a coordinated Mg^{2+} ion essential for binding nucleotide. Studies have shown that lowering the concentration of Mg^{2+} in solution, for example, by using a divalent chelator such as EDTA, dramatically increases the rate of nucleotide release from G-proteins [25]. In this manner, addition of EDTA can serve as an artificial GEF for GTPase activation. Hydrolysis of bound GTP to yield GDP and inorganic phosphate is carried out by a slow intrinsic activity that, in Ras, results in a half-life of about 20 min for bound GTP [25]. The universal switching mechanism of Ras superfamily GTPases stems from nucleotide-dependent conformational changes within two loop regions termed switch I and switch II [24]. GTP-bound protein is in an active conformation that binds downstream effector proteins with high affinity, while GDP-bound protein is unable to interact effectively with downstream targets.

Assays for G-Protein Function: Background

In the past, most methods for studying the activation of Ras members have relied upon radioactive forms of guanine nucleotides [26-28]. Typically in these assays, free nucleotides and inorganic phosphate are separated from nucleotide-bound forms of GTPases using differential binding to filters or activated charcoal. Unfortunately, all such methods suffer from several intrinsic disadvantages arising from the need to separate bound and free nucleotides. These disadvantages include extensive manual manipulations, limited data collection rates, low intrinsic precision, the production of radioactive waste, and

discontinuous monitoring of reaction kinetics. Furthermore, the physical separation of reactants introduces potential anomalies resulting from perturbations in reactant concentrations and the possible destabilization of native proteins.

Fortunately, recent advances in spectroscopic instrumentation and the production of a variety of fluorescent analogs of guanine nucleotides have enabled fluorescence-based assays, similar to those refined by Alfred Wittinghofer and colleagues [29], to become preeminent for studying many biochemical properties of G-proteins. These assays take advantage of spectroscopic differences between bound and unbound fluorescent analogs of guanine to monitor the binding and hydrolysis of nucleotides as well as the interaction of GTPases with various effectors and regulators. The most common analogs possess either BODIPY [30] or mant (N-methylanthraniloyl) [31] fluorophores covalently attached to the sugar hydroxyls of GDP, GTP, or non-hydrolysable forms of GTP such as 5'-[β,γ-imido]triphosphate (Gpp(NH)p). Such nucleotide analogs are used to study GTPases of the Ras superfamily [32] as well as heterotrimeric G-proteins [33-35] as described in the accompanying review by Kimple et al. in this issue of CCHTS.

Fluorophores emit light at a longer wavelength (λ_{em}) when excited at a certain wavelength (λ_{ex}). For example, excitation of the mant fluorophore at 360nm will result in a fluorescence emission at 440nm. A fluorophore-conjugated nucleotide has a low quantum yield of fluorescence in solution due to intermolecular quenching by solvent and intramolecular quenching by the guanine base. However, upon binding to G-protein, the fluorescence emission intensity from the fluorophore is greatly enhanced as illustrated in Fig. (2 a). The use of mant-nucleotides for the

study of Ras was first described by Neal et al. [32]. This study showed that binding mant-GTP to Ras results in an increase in fluorescence intensity of about 3.2-fold.

Although alternative fluorophores can be used to study small G-protein function, the mant fluorophore, shown conjugated to GTP in Fig. (2 b), is ideal for use due to its compact size and will be the predominant fluorophore discussed in this review. Mant nucleotides can be readily obtained commercially from Molecular Probes Inc. or large quantities can be synthesized in-house as initially described by Hiratsuka [31, 36]. The X-ray crystal structure of Ras bound to a mant-derivatized, non-hydrolysable analog of GTP revealed that the conjugated mant fluorophore does not significantly affect nucleotide binding nor impair interactions with effector proteins [37].
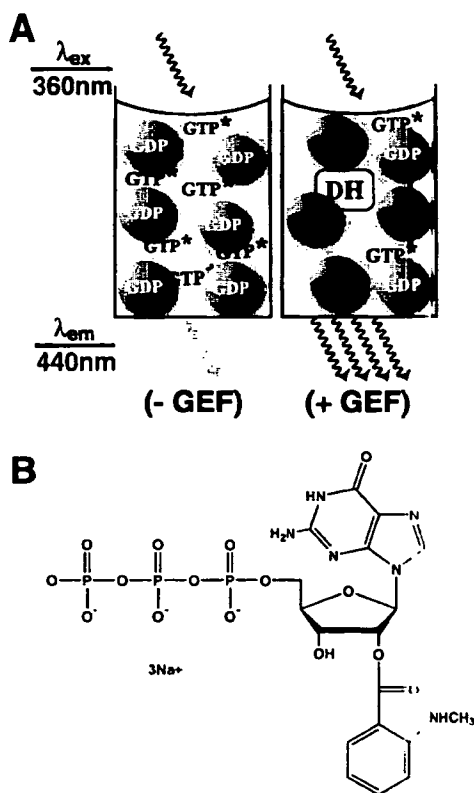
Fig. (2). Schematic overview of the fluorescent nucleotide exchange assay using mant-GTP. (A) Fluorescent nucleotides such as mant-GTP can be used as probes for small GTPase activation. Excitation of the mant fluorophore at 360 nm (λ_{ex}) will result in an increase in fluorescence emission at 440 nm (λ_{em}) when nucleotide is bound to G-protein. Addition of a GEF catalyzes the loading of mant-GTP onto the GTPase, thereby increasing the fluorescence emission. (B) Chemical structure of mant-GTP. Please note, mant-GTP isomerizes between the 2' and 3' sugar hydroxyl.

(1) FLUORESCENCE-BASED ASSAYS OF GTPASE ACTIVATION

GEFs for specific Ras subfamilies activate GTPases and share a high degree of sequence homology as well as

structural similarity. For example, GEFs specific for the Ras subfamily (Sos1/2, CDC25) are characterized by the presence of a CDC25 homology domain [38], while GEFs specific for the Rho subfamily (Vav, Dbl, Tiam1, Dbs) contain tandem Dbl homology (DH) and pleckstrin homology (PH) domains [16]. There is not a one-to-one concordance in the pairings between GTPases and GEFs such that some GEFs will activate numerous related GTPases while other GEFs are highly specific for individual GTPases. However the structural elucidation of several GEF-GTPase complexes has revealed a conserved mechanism of G-protein activation [38-42].

During the guanine nucleotide exchange cycle, GEFs function to stabilize the nucleotide-free state of the G-protein by binding to the inactive GDP-bound form. Once bound, the GEF catalyzes the expulsion of GDP and Mg^{2+}. In the cell, levels of GTP are much higher than GDP (~10-fold) and nucleotide-free G-proteins rapidly bind GTP leading to the restructuring of switches I and II associated with G-protein activation and the concomitant release of activating GEF. Since most G-proteins have similar affinities for the various guanine nucleotides and addition of fluorophores to the sugar hydroxyls does not typically alter these affinities [29], GEFs generally catalyze the loading of GDP, GTP, or Gpp(NH)p, as well as their fluorophore-conjugated analogs, with similar efficiencies.

Standard Assays of Nucleotide Exchange

The simplest fluorescence-based assay of nucleotide exchange is straightforward, rapid, and typically sufficient for monitoring GTPase activation in real-time. This assay is useful for assessing the competence of GTPases to bind guanine nucleotides in the presence of limiting concentrations of Mg^{2+} as well as for the biochemical characterization of GEFs. In this assay, a candidate GEF (or EDTA) catalyzes the expulsion of nucleotide from the small GTPase. Then, depending on which fluorescent nucleotide is predominant in solution, the fluorophore-conjugated nucleotide binds to the GTPase thereby causing an increase in the fluorescent signal. Fluorophores conjugated to both GDP and GTP can be used for this assay, as they will both bind to nucleotide-free G-protein. The GTPase is typically purified in a buffer containing GDP, to ensure protein stability.

A typical example of a fluorescent nucleotide exchange assay incorporating mant-GTP is pictured in Fig. (3). This figure shows Tiam1 activating the Rho subfamily member Rac1 [42]. Experimental conditions for this particular result are typical for fluorescent nucleotide exchange assays and can be applied to most studies of small GTPase activation, as well as other assays discussed in this review. Rac1 was purified in the presence of GDP and the catalytic DH/PH fragment of Tiam1 was used as an exchange factor. Typical reaction conditions are: 10% glycerol (v/v), 50 μg/mL bovine serum albumin (BSA), 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 400 nM mant-GTP, 200 nM GEF (Tiam1), and 2 μM GTPase (Rac1) in 1 mL total volume. For our example of Tiam1 activation of Rac1, a reaction containing all reagents except for Tiam1 was allowed to equilibrate for ~500 seconds in a
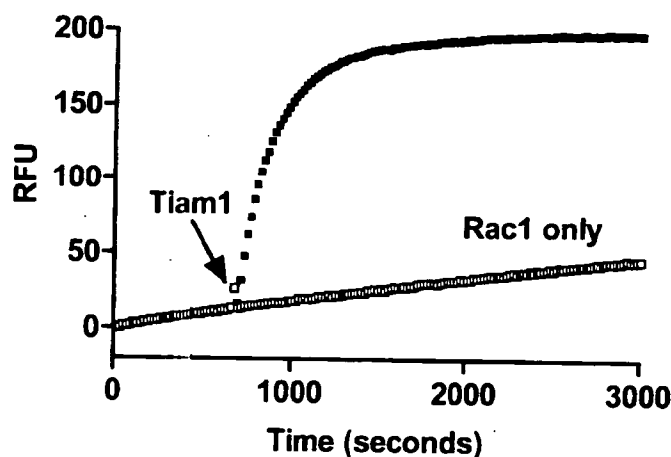
Fig. (3). Fluorescent nucleotide exchange assay. 2 µM Rac1 was incubated either in the presence (■) or absence (□) of the Rac1-specific RhoGEF Tiam1 (200 nM). Exchange wa: initiated by addition of Tiam1 as indicated and monitored using the fluorescent nucleotide exchange assay with mant-GTP [42].

1500 µL thermo-statted cuvette (25° C) with constant stirring. After this initial equilibration, Tiam1 was manually injected into the mixture to initiate the exchange reaction. Fluorescence emission was then monitored until completion using a Perkin-Elmer LS-55 fluorimeter with the following settings: λ_{ex} = 360 nm, λ_{em} = 440 nm, slits = 5 nm.

While this simple fluorescent nucleotide exchange assay is sufficient to describe relative levels of GTPase activation in a format that is readily applicable to most GTPases, it is not sensitive enough to determine catalytic rates of reaction. The major problem is that fluorescent nucleotide reactants in solution are typically in limiting quantities, i.e. only 400 nM. This dilemma can easily be circumvented by increasing the concentration of fluorescent nucleotides in solution to non-limiting quantities (~100 µM). However, given the cost of purchasing or synthesizing large quantities of fluorescent nucleotides, this remedy is often impractical. Consequently,

kinetic data is typically collected using G-proteins preloaded with fluorescent guanine nucleotides as described in the next section.

Assays of Nucleotide Exchange Using Preloaded GTPases

In this assay, G-protein is preloaded with fluorescent nucleotide and the exchange reaction is carried out in a solution containing an excess of non-fluorescent guanine nucleotide. The main use for this assay is for kinetic analysis of guanine nucleotide dissociation rates and can be extended to the study of GEFs, GAPs, GDIs, and effectors.

For example, using Cdc42 preloaded with mant-GDP we showed that the Cdc42(C18A) mutant functions as a dominant negative during the guanine nucleotide exchange reaction as illustrated in Fig. (4) [43]. In this experiment,
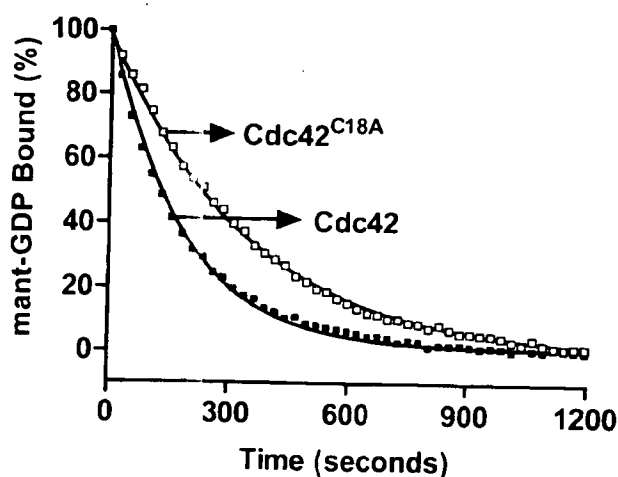
Fig. (4). Preloaded GTPase nucleotide exchange assay. 400 nM of mant-GDP preloaded wild type Cdc42 was incubated with 2 µM of either wild type Cdc42 (■) or mutant Cdc42(C18A) (□). Guanine nucleotide exchange was initiated by addition of 200 nM Dbs in the presence of 20 µM GDP. The Cdc42(C18A) mutant acts as a dominant negative, slowing the rate of nucleotide exchange by Dbs on wild type Cdc42 [43]. Curves were fit as single exponential decay functions using GraphPad Prism (GraphPad Software, Inc.).

purified wild-type Cdc42 was preloaded with mant-GDP by incubation in a solution containing ~2-fold molar excess mant-GDP, 5 mM EDTA, 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 5% glycerol (v/v). After 30 minutes at room temperature, $MgCl_2$ was added to a final concentration of 20 mM to terminate nucleotide exchange and Cdc42 was subsequently exchanged into buffer containing 20 mM Tris (pH 7.5), 50 mM NaCl, and 5 mM $MgCl_2$ using gel exclusion chromatography. The protein was then used for a standard guanine nucleotide exchange assay in a solution consisting of 400 nM mant-GDP preloaded Cdc42, 200 nM Dbs (GEF), 20 μM GDP, and 2 μM of either wild type Cdc42 or mutant Cdc42[C18A]. As shown in Fig. (4), the presence of the Cdc42[C18A] mutant slows the ability of Dbs to catalyze release of mant-GDP from wild type Cdc42. This Cdc42[C18A] mutant was subsequently shown to cause a decrease in the G-protein's affinity for guanine nucleotide, resulting in the sequestration of Dbs in a stable complex with Cdc42[C18A] [13].

FRET-Based Assays of Nucleotide Exchange

Assays of nucleotide exchange based upon fluorescence resonance energy transfer (FRET) typically rely upon the intrinsic fluorescence emission from tryptophan residues within the GTPase. Tryptophan residues have environmentally sensitive spectroscopic properties resulting in a steady-state fluorescence emission maximum at ~335 nm when excited at ~295 nm. Biochemists have taken advantage of this unique spectroscopic property of tryptophan for some time to study structure and folding in a wide variety of proteins [44]. Traditional approaches typically monitor changes in the fluorescence emission spectra of tryptophan residues upon changes in conformation within the protein; as the microenvironment of the tryptophan residue changes, so does its associated fluorescence spectrum.

However, unlike traditional intrinsic tryptophan fluorescence assays that directly monitor tryptophan fluorescence, typical FRET-based assays rely upon the non-radiative transfer of energy between ultraviolet light-excited tryptophans within the protein under study and nearby fluorophores having overlapping energy spectra [45]. This energy transfer is extremely sensitive to the distance separating the two fluorophores and has been used to study protein-protein interactions as well as conformational changes in numerous proteins. In a typical nucleotide exchange assay relying on FRET, fluorescence energy from a donor tryptophan can excite an acceptor mant fluorophore. This energy transfer is observed only when fluorescent nucleotide binds to G-protein and can thereby be used to quantify the amount of nucleotide-bound G-protein. FRET between tryptophan and mant-nucleotide caused by exciting tryptophan ($\lambda_{ex} = 295$ nm) results in two useful, reciprocal changes in the fluorescence spectra: (1) emission ($\lambda_{em} = 440$ nm) of the mant fluorophore increases while (2) emission ($\lambda_{em} = 335$ nm) from tryptophan decreases.

Monitoring increases in mant fluorescence due to energy transfer from excited tryptophan is an alternative approach to directly exciting the mant fluorophore. The benefit of using this approach is an increase in the signal to noise ratio, as exemplified in Fig. (5) showing the spontaneous loading of mant-GTP onto the Ras-related protein Rap2A. In this example, fluorescence emission ($\lambda_{em} = 440$ nm) of bound mant-GTP via FRET upon excitation of tryptophan ($\lambda_{ex} = 295$ nm) leads to approximately a 2-fold increase in the signal-to-noise relative to direct excitation of the mant fluorophore ($\lambda_{ex} = 360$ nm).

Although FRET-based approaches to studying small GTPase function are extremely sensitive, the requirement for at least one tryptophan residue precludes the study of certain G-proteins, such as Ras, that lack tryptophan. Nevertheless, strategic introduction of a single tryptophan residue using
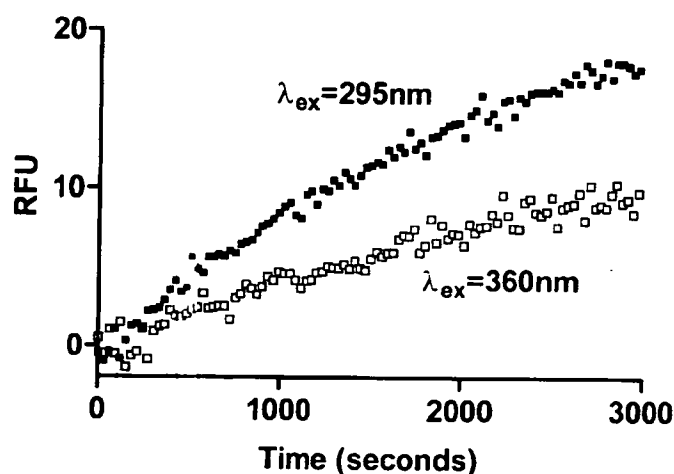

Fig. (5). Comparison of direct excitation of the mant fluorophore vs. FRET-based excitation by tryptophan. The intrinsic nucleotide exchange rate of 100 nM Rap2A was monitored using the fluorescent nucleotide exchange assay with mant-GTP in the absence of a RapGEF. Intrinsic exchange of Rap2A was monitored by either direct excitation of the mant fluorophore at 360 nm (□), or indirect excitation of tryptophan within the GTPase at 295 nm (■). Fluorescence emission of the mant fluorophore was then measured at 440 nm. Excitation of tryptophan results in a FRET-based excitation of bound mant-GTP and yields ~2-fold increase of the signal to noise ratio observed compared to excitation of the mant group directly.

site-directed mutagenesis has been shown sufficient to allow fluorescence-based studies without perturbing the G-protein's activity [46, 47]. Furthermore, FRET-based assays are particularly useful for studying the activation of the Rho subfamily GTPases, due to the presence of a strictly conserved tryptophan residue.

Fig. (6) illustrates the use of FRET for the biochemical characterization of GEFs responsible for the activation of Rho GTPases [41]. As shown in Fig. (6), a single point mutation (Y889F) within the pleckstrin homology (PH) domain of Dbs (Dbl's big sister) is sufficient to abolish the exchange activity of Dbs on both RhoA and Cdc42. In this example, rather than monitoring an increase in mant fluorescence as shown in Fig. (5), we monitored a decrease in tryptophan fluorescence resulting from FRET. Tryptophan within RhoA or Cdc42 was excited at 295 nm and steady-state fluorescence was monitored at 335 nm in a buffer initially lacking fluorescent nucleotide and consisting of: 10% glycerol, 50 μg/mL BSA, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, 10 mM MgCl$_2$, 200 nM Dbs (GEF), and 2 μM RhoA or Cdc42 (GTPase). After a period

Fig. (6). Tryptophan FRET assay. The Y889F mutation (▲) within the PH domain of Dbs impairs guanine nucleotide exchange activity towards RhoA (A) and Cdc42 (B) compared to wild type Dbs (■) or no Dbs present (□). Fluorescence emission of tryptophan at 335 nm was monitored by excitation at 295 nm. Dbs catalyzed loading of mant-GTP onto GTPase causes a decrease in the fluorescence emission of tryptophan due to FRET between bound mant-GTP and intrinsic tryptophan [41]. Curves were fit as single exponential decay functions using GraphPad Prism (GraphPad Software, Inc.).

of equilibration, the exchange reaction was initiated by the addition of mant-GTP. RhoA and Cdc42 used for this example were purified in the presence of GDP; therefore, the observed steady-state tryptophan fluorescence in the absence of mant-GTP correlates with 100% GDP-bound. Once the reaction is initiated, Dbs catalyzes loading of mant-GTP until 100% of the GTPase is GTP-bound.
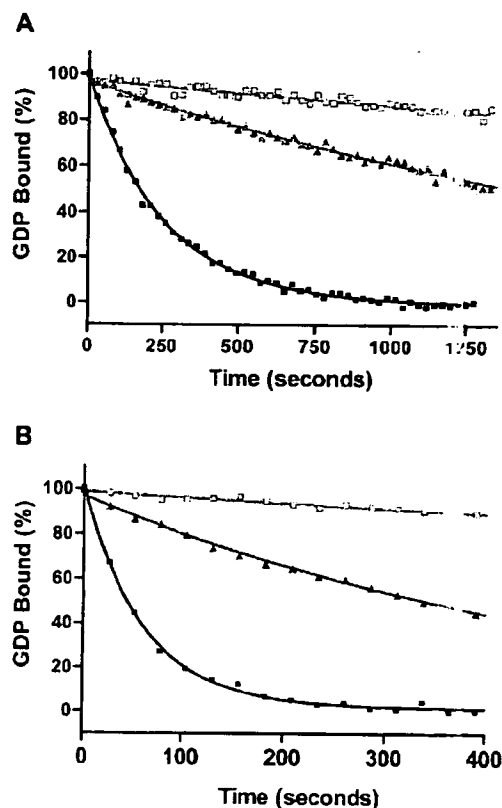
Emerging FRET-Based Techniques for Live Cell Imaging

The study of spatial-temporal activation of Ras superfamily GTPases using FRET in living cells is an emerging field in G-protein research. Studies devoted to live cell imaging describe activation and localization of GTPases in response to various stimuli using biosensors that are sensitive to GTPase activation and subsequent interaction with downstream effectors (for review see [48]).

Within the cell, Ras GTPases are modified by lipid groups at their C-termini, resulting in localization to the inner membranes of cells. Upon appropriate stimulation, membrane-associated GTPases are activated by GEFs, allowing further downstream interactions with effector proteins generally localized to the plasma membrane. Stimuli, such as a chemoattractant gradient, can induce altered distributions of activated G-protein, resulting in gradients that allow cell polarization. This is especially evident in the case of Rho subfamily GTPases during actin cytoskeletal rearrangement in neutrophils [49]. Emerging techniques using FRET have allowed investigators to study the highly coordinated events involved in Ras GTPase activation [50, 51]. Although still in its infancy, these fluorescence-based techniques for imaging G-protein activation have been instrumental in defining the molecular physiology of small GTPases in the context of living cells.

The use of FRET for real-time live cell imaging of small GTPase activation has been described for Rac [52-54], Ran [55, 56], Cdc42 [57], as well as Ras and Rap [58]. In each case, activated GTPase is detected by the expression of one or more biosensors. Biosensors typically contain two fluorophores that can interact to yield FRET-based changes in fluorescence emission due to intra- or inter-molecular interactions that vary upon interaction with active GTPases [48]. Alternatively, FRET can be measured between an effector binding domain conjugated to a fluorescent probe and a GTPase fused to green fluorescent protein (GFP) or its variants [50, 51].

For example, Kraynov et al. monitored activation of Rac fused to GFP by measuring FRET upon interaction with the Rac-binding domain of p21-activating kinase (Pak1) derivatized with AlexaFluor 546 [52]. The authors used this strategy to show that PDGF stimulation induces moving ruffles of FRET associated with Rac activation and actin polymerization [52]. Similarly, in studies of the nuclear GTPase Ran, Kalab et al. expressed a biosensor consisting of the effector binding domain from the yeast Ran-GAP accessory factor Yrb1 separating yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) [55]. In the absence of GEF, Ran exists in the inactive GDP-bound form and does not interact with the Ran binding domain so that YFP and CFP are in close proximity to facilitate FRET.

However, upon co-expression with a RanGEF, such as RCC1, the FRET signal is reduced as binding of Ran to the Ran binding domain disrupts the interaction between the two fluorophores. The authors used this approach to visualize nuclear Ran activation gradients during interphase [55].

(2) FLUORESCENCE-BASED ASSAYS OF EFFECTOR COUPLING

Effector proteins for Ras superfamily GTPases preferentially interact with the GTP-bound form of the G-protein and function to further propagate signal transduction. Ras has a large number of effector proteins; however, the most characterized downstream targets in Ras-mediated transformation are Raf kinase, RalGDS, and phosphotidylinositol-3-kinase (PI3-K) [2]. Likewise, Rho GTPases have multiple downstream effector proteins (over 20) involved in actin cytoskeletal regulation including p21-activated kinase (Pak), Wiskott-Aldrich syndrome proteins (WASP), Rho-associated kinase (ROCK), and activated Cdc42-associated kinase (Ack) [6].

Effector proteins for Ras superfamily members are typically characterized by the presence of a binding domain with high sequence homology that is essential for interaction with the GTPase. Binding domains for Ras subfamily effectors, are termed the "Ras binding domain" (RBD) and "Ras-association" (RA) domain. In the case of Rho effectors, this domain is generally referred to as a "GTPase binding domain" (GBD) and often is made up of a conserved sequence termed the Cdc42/Rac-interactive binding (CRIB) domain or the p21 binding domain (PBD). The binding interface between Ras or Rho and their associated effector proteins has been extensively characterized through structural studies including the crystal structures of RapIA-Raf [59], and Cdc42-Pak [60]. These studies and others have shown

that GTP-dependent conformations within switch I and II of the GTPase determine effector specificity.

While techniques such as the yeast two-hybrid screen have allowed for discovery of novel effectors for Ras superfamily GTPases, fluorescence-based assays have been instrumental in the biochemical analysis of effector coupling and determination of binding affinities. Furthermore, emerging techniques are currently being developed that utilize FRET to characterize effector binding.

Inhibition of Guanine Nucleotide Dissociation by Effector Coupling

Complexes between activated GTPases and effector proteins are typically stable enough to dramatically decrease the intrinsic guanine exchange cycle of the G-protein. This property of effector proteins has lead to the development of assays that correlate guanine nucleotide dissociation rates with binding affinities for GTPase effectors that have been used in the past to determine binding affinity constants between Ras superfamily GTPases and their effectors [61-63].

An example illustrating inhibition of GTP release upon interaction with effectors is highlighted in Fig. (7) for the small GTPase Rap2A interacting with the tandem RBDs of RGS14 [64, 65]. In this example, 1 mL cuvettes were loaded with 1 μM mant-GTP (in 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA at 37° C) and allowed to equilibrate prior to the addition of 100 nM Rap2A (preloaded with Gpp(NH)p) in the presence or absence of 1 μM of the RGS14 RBD protein. We then measured dissociation of Gpp(NH)p by excitation of the tryptophan residue and measured FRET-mediated excitation of bound mant-GTP. As shown in Fig. (7), incubation of Rap2A with the RGS14 tandem RBD repeat region slowed the rate of spontaneous nucleotide exchange by ~30% (p < 0.05)
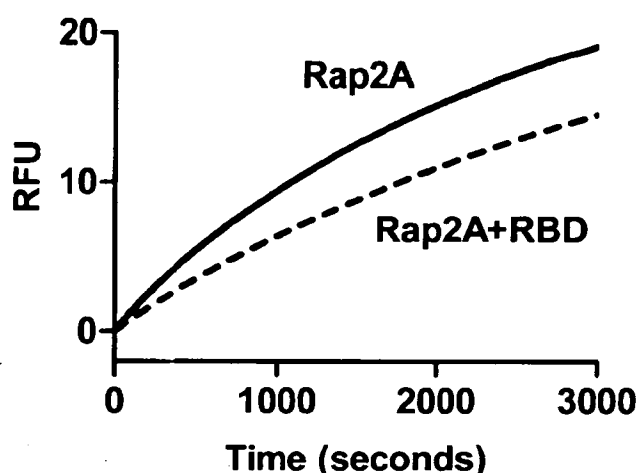
Fig. (7). GDI assay for effector coupling. The intrinsic guanine nucleotide exchange rate of Gpp(NH)p-preloaded Rap2A (100 nM) was monitored with mant-GTP in the absence (solid black) or presence (dashed gray) of the RBD tandem repeat (aa 300-447) from the putative Rap2A effector rat RGS14 (1 μM). Binding of RGS14 RBD repeat region to activated Rap2A prevents the loss of guanine nucleotide from the G-protein, thereby effectively acting as a guanine nucleotide dissociation inhibitor (GDI). Addition of RGS14 RBD repeat region protein slows the initial rate of intrinsic exchange by ~30% (p < 0.05) compared to that of Rap2A alone. Curves were fit as single exponential functions using GraphPad Prism (GraphPad Software, Inc.).

compared to that of Rap2A alone. Previous studies incorporating this strategy were instrumental in determining binding affinities of Ras with the RBD of Raf and RalGDS [61, 62] and was later applied to biochemical characterization of Ran with its effector RanBP [63].

Emerging FRET-Based Assays for Effector Coupling

FRET-based assays for effector coupling utilize fluorescent biosensors similar to those used for live cell imaging of G-protein activation. In many cases, the same biosensors expressed in cells can be affinity purified and used for *in vitro* testing [52, 55]. Graham et al. have developed a FRET-based assay for detecting Rac and Cdc42 interactions with effector proteins [66]. Their biosensor consists of the CRIB domain of Pak flanked by an N-terminal green fluorescence protein (GFP) and a C-terminal blue fluorescence protein (BFP), a strategy similar to that described by Kalab et al. for the study of Ran activation [55]. Activated G-protein binds to the CRIB domain, thereby separating the two fluorophores and reducing the amount of FRET observed. This Cdc42/Rac1 biosensor can be used for *in vitro* analysis of effector coupling. For example, a putative effector for Cdc42 or Rac could be tested by incubating the candidate protein with the biosensor and GTPase. A true effector would compete with the biosensor for binding of G-protein, thereby increasing the amount of FRET signal from the biosensor.

(3) HIGH-THROUGHPUT APPLICATIONS

A major advantage of using a fluorescence-based assay for studying GTPase activation is the ability to format the assay for flexible, high-throughput applications. In particular, the fluorescent nucleotide exchange assay can be easily reformatted for use with fluorimeters designed to accept microtiter plates, thereby allowing large number of samples to be assayed in real-time. All previous examples listed above of fluorescence-based techniques were low-

throughput, as they were carried out using a fluorimeter that allows for only four reactions to occur simultaneously, each with a minimum volume of 1 mL. Although extremely sensitive, this setup is less than ideal for large-scale screening efforts since a single reaction will use up to 400 µg of GTPase and 10 µg of GEF. Redesigning the assay for a 96-well formatted fluorimeter allows up to 96 reactions to be monitored concurrently for G-protein activity, each with a total volume of only 100 µL. Thus, the major benefit of using a 96-well formatted assay for G-protein function is the ability to run multiple reactions while consuming much lower quantities of protein on a per sample basis.

96-Well Formatted Fluorescent Nucleotide Exchange Assay

Our group utilizes a 96-well formatted mant-GTP exchange assay for a number of applications, including biochemical characterization of Ras-superfamily GTPases and their associated GEFs. This format allows us to screen a large number of mutant protein constructs for activity and substrate specificity. Multiple truncation mutants of GEFs or point mutations of G-proteins can be readily assayed simultaneously. We are currently using this approach to analyze the substrate specificity of novel RhoGEFs against a panel of over 20 Rho GTPases. With this assay, we are also able to determine the activity of newly characterized RhoGEFs and explore regulatory elements within these proteins.

Additionally, we are currently using a high-throughput version of the fluorescent nucleotide exchange assay for target-based drug discovery of novel small molecule inhibitors of Rho GTPase activation. Complementing our ongoing structure-based drug discovery efforts, our group routinely screens large libraries of plated compounds using a 96-well formatted mant-GTP exchange assay. RhoGEF inhibitors identified using this strategy will help to further understand the physiological role of Rho GTPase signaling and could lead to future drugs for the treatment of metastatic
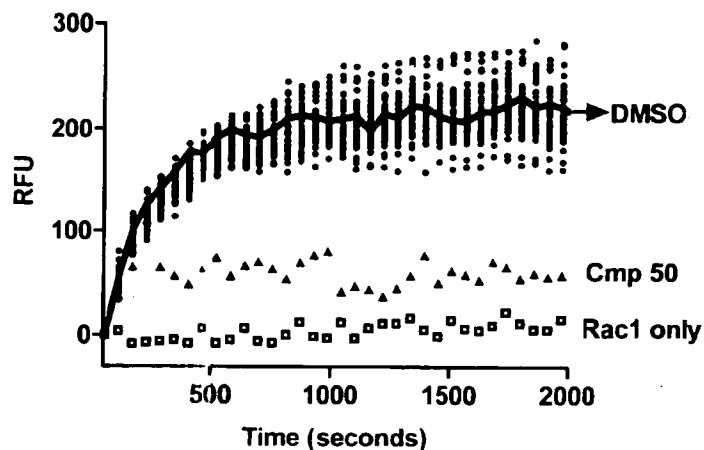

Fig. (8). 96-well formatted fluorescent nucleotide exchange assay. 50 compounds were screened for inhibition of Tiam1-mediated activation of Rac1 using a high-throughput fluorescent nucleotide exchange assay with mant-GTP. Compounds 1-49 (●) do not inhibit exchange compared to DMSO (⊡), while compound 50 (100 µM) (▲) inhibits Tiam1-catalyzed exchange of Rac1 similar to that of no Tiam1 present (□).

cancer. An example using this assay to screen a large number of compounds for inhibition of Tiam1-mediated Rac1 activation is shown in Fig. (8). In this example, 50 compounds were screened simultaneously for their ability to disrupt Tiam1 activation of Rac1. Although conditions for the 96-well formatted version of the mant-GTP assay are currently being refined, the assay can nevertheless effectively monitor GEF-induced GTP loading onto a variety of Ras-superfamily GTPases. A goal within our lab is to adapt many of the above-mentioned fluorescence-based assays for small G-protein function to use in a high-throughput manner.

ACKNOWLEDGEMENTS

ABBREVIATIONS

CRIB	=	Cdc42/Rac-interactive binding domain domain
Dbl	=	diffuse B-cell lymphoma
Dbs	=	Dbl's big sister
DH	=	Dbl homology
DTT	=	dithiothreitol
EDTA	=	ethylenediamine tetra-acetic acid
FRET	=	fluorescence resonance energy transfer
G-protein	=	guanine nucleotide binding protein or GTPase
GAP	=	GTPase activating protein
GDI	=	guanine nucleotide dissociation inhibitor
GEF	=	guanine nucleotide exchange factor
LARG	=	leukemia-associated RhoGEF
mant	=	N-methylanthraniloyl
PDGF	=	platelet-derived growth factor
PH	=	pleckstrin homology
RA	=	Ras-association
RBD	=	Ras binding domain
RhoGEF	=	Rho subfamily-specific GEF
Tiam1	=	T-cell lymphoma invasion and metastasis factor-1

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What is claimed is:

1.   A method of identifying a compound having the ability to modulate the guanine nucleotide exchange cycle of Ras superfamily GTPases, comprising:
        a) contacting the compound with a guanine nucleotide exchange factor and a GTPase and obtaining a baseline fluorescence measurement;
        b) contacting the guanine nucleotide exchange factor and the GTPase without the compound and obtaining a baseline fluorescence measurement.;
        c) adding a fluorophore-conjugated GTP to the components of (a) and (b), respectively;
        d) obtaining fluorescence measurements of the components of (c) over time;
        e) subtracting the baseline fluorescence measurements of (a) and (b) from each fluorescence measurement of (d); and
        f) comparing the resulting fluorescence values of (e), wherein a decrease or increase in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound having the ability to modulate the guanine nucleotide exchange cycle of Ras superfamily GTPases.

2.   A method of identifying a compound having the ability to inhibit guanine nucleotide exchange factor activity, comprising:
        a) contacting the compound with a first guanine nucleotide exchange factor and a GTPase and obtaining a baseline fluorescence measurement;
        b) contacting the first guanine nucleotide exchange factor and the GTPase without the compound and obtaining a baseline fluorescence measurement.;
        c) adding a fluorophore-conjugated GTP to the components of (a) and (b), respectively;
        d) obtaining fluorescence measurements of the components of (c) over time;
        e) subtracting the baseline fluorescence measurements of (a) and (b) from the fluorescence measurements of (d);
        f) comparing the resulting fluorescence values of (e), wherein a decrease in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound potentially having the ability to inhibit guanine nucleotide exchange factor activity;
        g) repeating steps a-e with a second guanine nucleotide exchange factor;
        h) comparing the resulting fluorescence values of (g), wherein no decrease in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound having the ability to inhibit guanine exchange factor activity.

3.   A method of identifying a compound having the ability to inhibit GTPase activity, comprising:
        a) contacting the compound with a guanine nucleotide exchange factor and a first GTPase and obtaining a baseline fluorescence measurement;
        b) contacting the guanine nucleotide exchange factor and the first GTPase without the compound and obtaining a baseline fluorescence measurement;

    c) adding a fluorophore-conjugated GTP to the components of (a) and (b), respectively;

    d) obtaining fluorescence measurements of the components of (c) over time;

    e) subtracting the baseline fluorescence measurements of (a) and (b) from the fluorescence measurements of (d);

    f) comparing the resulting fluorescence values of (e), wherein a decrease in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound potentially having the ability to inhibit guanine nucleotide exchange factor activity;

    g) repeating steps a-e with a second GTPase; and

    h) comparing the resulting fluorescence values of (g), wherein no decrease in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound having the ability to inhibit GTPase activity.

4.   A method of identifying a compound having the ability to modulate effector/GTPase activity, comprising:

    a) contacting the compound with a GTPase and an effector protein and obtaining a baseline fluorescence measurement;

    b) obtaining a baseline fluorescence measurement of the GTPase and the effector protein without the compound;

    c) adding a fluorophore-conjugated GTP to the components of (a) and (b), respectively;

    d) obtaining fluorescence measurements of the components of (c) over time;

    e) subtracting the baseline fluorescence measurements of (a) and (b) from the fluorescence measurements of (d); and

    f) comparing the resulting fluorescence values of (e), wherein a decrease or increase in the rate of fluorescence change with the compound as compared with the rate of fluorescence change without the compound identifies a compound having the ability to modulate effector/GTPase activity.

5.   The method of any of claims 1-4, wherein the guanine nucleotide exchange factor is selected from the group consisting of Ect2, Bcr, Abr, RasGRF, Sos, Neuroblastoma, S-GEF, Vsm-RhoGEF, N-GEF,Tim, Intersectin, Xpln, Net1, LARG, p115-RhoGEF, PDZ-RhoGEF, Lfc, Lbc,p114-RhoGEF, Alsin, Tuba, P-Rex, Asef, Tiam1, Tiam2, alpha-Pix,beta-Pix, Dbs, Dbl, Trio, Duo, Duet, GEFT, Obscurin, Vav1, Vav2, Vav3, FGD1, Frabin, CDC25, ITSN, Sos1/2, any combination thereof and/or biologically active fragments or domains thereof.

6.   The methods of any of claims 1-4, wherein the GTPase is selected from the group consisting of H-Ras, N-Ras, R-Ras, K-Ras, Rap, Ral, Rab, Arf, Rad, Gem, Ran, RhoA, RhoB, RhoC, RhoD. RhoE, RhoF, RhoG, Cdc42, Rac1, Rac2, Rac3, TC10, TCL, Chp, Wrch, RhoBTB, any combination thereof and/or biologically active fragments or domains thereof.

7.   The method of claim 4, wherein the effector protein is selected from the group consisting of Pak, Rock, Raf, any combination thereof and/or biologically active fragments or domains thereof.

8.   A method of treating cancer in a subject, comprising administering to the subject an effective amount of a compound identified by an assay of this invention to modulate the guanine nucleotide exchange cycle of Ras superfamily GTPases; to inhibit guanine nucleotide exchange factor activity; to inhibit GTPase activity; and/or to modulate effector/GTPase activity.

9.   A pharmaceutical composition comprising a compound identified by an assay of this invention to modulate the guanine nucleotide exchange cycle of Ras superfamily GTPases; to inhibit guanine nucleotide exchange factor activity; to inhibit GTPase activity; and/or to modulate effector/GTPase activity, and a pharmaceutically acceptable carrier.
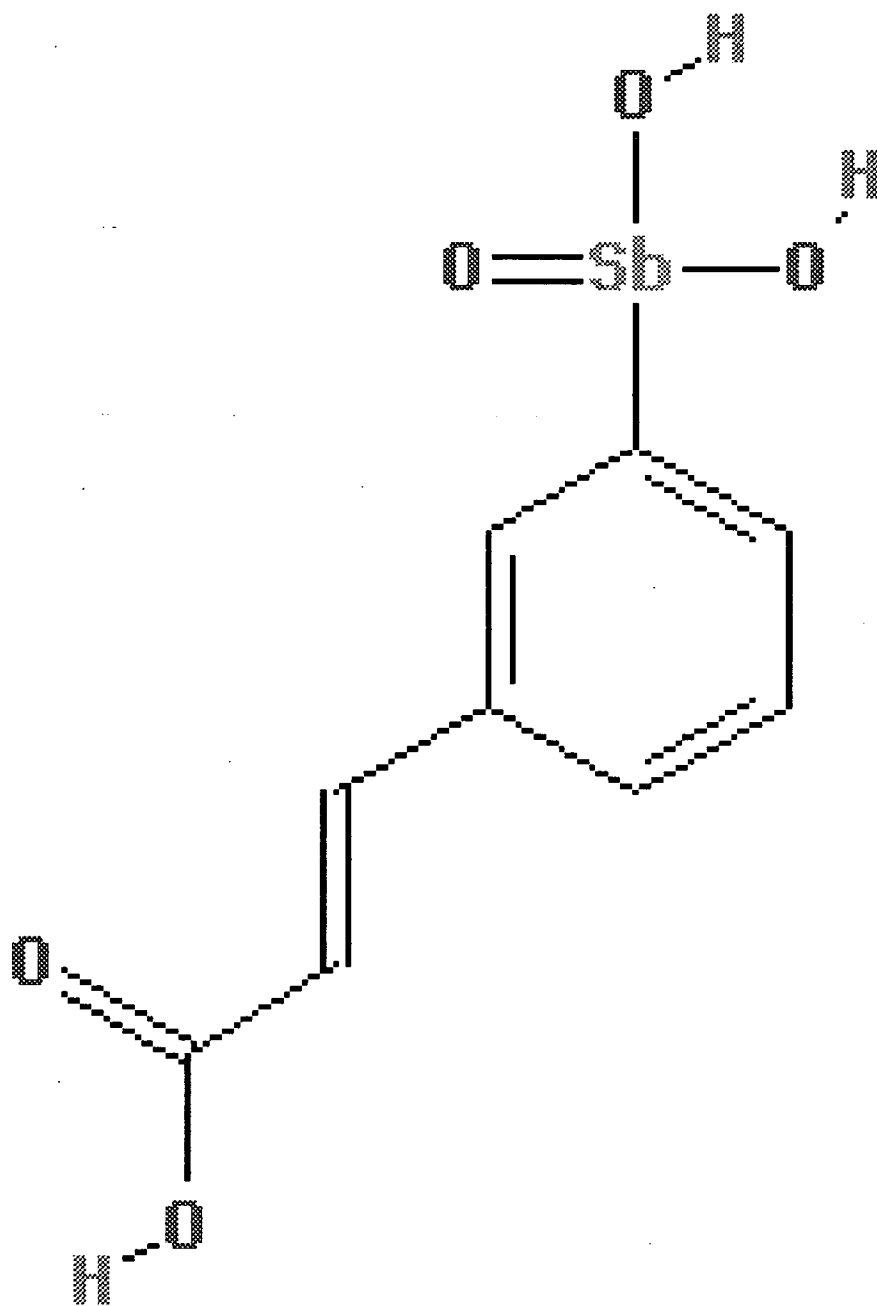
Figure 1